Pregnant Mares' Urine as a Source of Oestrogens

By W. G. Stevenson

PREGNANT mares' urine (P. M. U.) is an important commodity that has four to five times the monetary value of milk. The revenue derived from its sale represents an important source of income for horse-breeders, particularly since the present demand for work-horses is practically nil. Why and how P. M. U. has come to occupy this important position is an interesting story that had its beginning many years ago.

It has long been known that removal of the ovaries resulted in atrophy of the genitalia and cessation of reproductive activity. While Berthold (1) in 1849 showed that transplantation of the testes of a cockerel to other than normal locations of the body did not result in the physical changes characteristic of capons, it was not until about 1900 that Knauer (2) showed that the atrophy of the genitalia which follows ovariectomy did not occur in rabbits and guinea pigs when the ovaries were removed from the abdominal cavity and transplanted to other positions. This was definite proof that the ovary secreted a hormone that was responsible for the maintenance of the reproductive organs. Following this, several workers tried to demonstrate the presence of the chemical responsible for these effects in ovarian extracts; however, early attempts were not successful, and it was not until around 1912 that several workers were able to show that extracts of ovaries and human placenta when injected into suitable animals initiated changes characteristic of oestrus. Nevertheless, little headway was made in the isolation of the hormone responsible for these reactions. This was chiefly because tests for oestrogen were extremely cumbersome, highly technical and time-consuming, being based on the maintenance of uterine weight in castrated females, or the development of the uterus of immature females. Therefore, when Allen and Doisy (3) described a simple, rapid and inexpensive test based on the ability of oestrogens to induce changes typical of oestrus in the vaginal epithelial cells of castrated mice and rats, more rapid progress was made possible. Using the Allen-Doisy test, it is possible to detect one part of oestrogen in one billion parts of extraneous material.

Progress in the search for the active ovarian factor was also retarded because there was no really good source of oestrogen available. For instance, in the early work a favourite source of oestrogen was sows' ovaries; however, they were far from satisfactory as the concentration was extremely small, being only 6 mgs. of alpha estradiol and estrone respectively per ton of ovaries.

Although there were no really good sources of oestrogen known, due to the sensitivity of the Allen-Doisy test many different tissues or secretions, such as ovaries, corpora lutea, liquor folliculi, human, bovine and ovine placentae, blood, bile, feces, testes and even vegetables, such as po-

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tatoes, rape seed, yeast, pussy willows and palm kernels, were shown to possess oestrogenic activity. Still, progress was slow, and it was not until the discovery of Ascheim and Zondek (3) in 1927 that the urine of pregnant women was a rich source of readily available oestrogen that rapid advance was made. Within two years of this observation, Doisy (5), Butenandt (6)and Laqueur (7), working independently, isolated crystalline oestrone from human pregnancy urine.

In 1930 Collip (8) reported the presence of a new type of oestrogenic hormone in human placenta, to which he gave the name of "Emmenin". This differed from the other oestrogens known at that time by being very soluble in water and highly active when administrated orally. Later Collip's group established that the oestrogens of human pregnancy urine occur naturally in a water soluble, ether insoluble, orally active form, and their identity with "Emmenin" obtained from human placenta was proven.

Kober and Marian (9) in 1936 isolated the principal oestrogen of human pregnancy urine and identified it as estriol glycuronide. Preparations containing oestrone or estriol glycuronide were available soon for clinical use. Although every woman over forty is a potential user of oestrogens, their use was limited. Several factors were responsible but the difficulties associated with the large scale collection of human pregnancy urine, resulting in limited supplies and high cost, were important. For obvious reasons, only women in low income groups are interested in collecting urine and as soon as other sources of income are available and their economic position improves, they cease collecting. This has been forcibly demonstrated during the war years when the quantity of human pregnancy urine collected has been markedly diminished.

Before large supplies of oestrogens could be prepared at a price the average woman could afford to pay, it was necessary to obtain some source of material that was available in large quantities, and from which the cost of extracting the oestrogen was relatively low. Thus, Zondek's (10) observation that the urine of pregnant mares was a rich source of oestrogenic substances was most timely, and made possible the large scale commercial preparation of oestrogen.

Intensive study indicated that P. M. U. contained oestrone and other active oestrogens, such as equilin, equilenim, alpha estradiol, alpha dehydro-equilenim, beta estradiol and hippulin. However, its oestrogenic effect is largely due to oestrone as the concentration of the other oestrogens is relatively low. Almost immediately after P. M. U. was recognized as a good source of oestrogen, products prepared from it were available commercially. A few years later, stallion urine was shown to be a good source of oestrogen, and oestrogenic products prepared from it were also made available.

Why stallions should excrete oestrogen has never been satisfactorily explained. However, it would appear that the oestrogen is secreted by the testicles, as Feiser (11) states "the testes of the horse is the richest tissue known containing oestrogenic hormone", and because oestrogen excretion ceases following castration.

We have frequently assayed stallion urine to ascertain if any correlation exists between the breeding efficiency of the animal and the excretion of oestrogen in the urine. To date, we have been unable to determine any such relationship.

Until 1942, all natural oestrogens prepared from mare or stallion urine consisted of free oestrone or mixtures of free oestrone, and other oestrogens, and consequently were relatively inactive when given orally, and had to be given parenterally to obtain the desired therapeutic response. This is a very serious drawback, as few women wish to be injected twice weekly for several months, or in the case of castrated women for years. While the water soluble oestrogen "Emmenin" obtained from human pregnancy urine was available, it was not sufficiently potent to control severe oestrogen deficiencies, so the search was continued to obtain a water soluble, orally active oestrogen of sufficient potency that it might be used for any condition in which oestrogens were indicated.

Observations made by Zondek (12) indicated that the oestrogens present in P. M. U. were actually present in a water soluble state but that these were rapidly hydrolized by acid of putrefactive processes to water insoluble, free oestrogens. However, many technical difficulties were encountered in attempting to isolate the water soluble conjugated oestrogens from mares' urine. After many attempts, Schacter and Marian (13) did succeed in isolating a small quantity of potassium oestrone sulphate in crystalline form from P. M. U., and, the following year oestrone sulphate was prepared synthetically by Butenandt and Hofstetter. (14)

Ayerst, McKenna & Harrison, Limited, in co-operation with the Department of Biochemistry of McGill University under the direction of Dr. J. B. Collip, undertook extensive researches to develop a process whereby the conjugated water soluble, orally active oestrogens of mares' urine could be produced for clinical use at a price that would enable their widespread use. Intensive study showed that the concentration of oestrogen in urine varied considerably from mare to mare, from day to day, and with different stages of gestation; however, on the average, sufficient oestrogen was present from about the 100th day after service to parturition to make it worthwhile to process. It was also found that many factors, such as temperature of the surroundings, humidity, quantity and kind of feed consumed, and even exercise, affected the quality of urine secreted. By 1941, sufficient progress had been made to commence commercial production of the potent, orally active, conjugated oestrogens of P. M. U.

Early attempts to procure suitable urine were not entirely satisfactory. Once it becomes known in a community that there is a good market for pregnant mares, all the mares in the community immediately seem to become pregnant, regardless of whether they have been bred. As the concentration of oestrogen in non-pregnant mares' urine is extremely low, mixing it with P. M. U. may bring the oestrogen content of the latter down to such a level that it is impractical to process it. Therefore, it was necessary to institute some method that would detect the pregnant and non-pregnant mares. Fortunately, sensitive tests for pregnancy were available. Briefly, these con-

sist of biological tests for the demonstration of equine gonadotrophic hormone during the early stages of pregnancy, that is from the 40th to the 120th day after coitus, and biological or chemical tests for the detection of oestrogen after the latter date. However, the biological tests, whether for gonadotrophin or oestrogen, are time-consuming and relatively expensive, particularly when a large percentage of the animals tested are not pregnant.

Cuboni (15) described a chemical test for pregnancy in mares based on the demonstration of oestrogen in their urine. Schramm (16) found Cuboni's test gave 94% correct results on 4264 tests conducted after the 120th day of pregnancy, while Svenson (17) observed only 2.8% error in a group of 142 mares tested by the same method. Kober (18), using his quantitive test for oestrogens in urine, found that of 2507 mares classified as pregnant only 16 were non-pregnant, while of 1090 mares classified as non-pregnant only 6 were pregnant. During the past two years, we have conducted over 10,000 chemical tests for pregnancy using the Kober test and a simplification of the Cuboni test, as described by Roth Mayer and Bogart (19) for diagnosis of pregnancy in swine. Because samples are received from dealers by mail from such distant districts as Alberta with no data whatsoever, it is impossible to keep absolutely accurate data. Furthermore, we do not know the percentage of false negatives that occur because mares that are classified as negative are not purchased. However, from questionnaires distributed to various dealers and producers, it would appear that the number of false negatives, that is, pregnant mares classified as non-pregnant, is probably not more than one percent when the test is conducted on or after the 140th day of the gestation period. The number of false positives, that is, non-pregnant mares that give a positive reaction, is much higher, in some units exceeding 10%. For instance, in one unit of 30 mares, four gave positive tests, and, although they were not observed to abort or foal, they gave negative tests several months later. Two mares in a unit of 23 behaved similarly, and, doubtless, others have occurred but have not been reported. During the months these six mares were observed, none of them showed any indications of oestrus.

One aged, grade Clydesdale mare, severely affected with heaves, excreted a particularly high concentration of oestrogen over an extended period, although she was definitely not pregnant. This suggested the possibility of heaves influencing oestrogen excretion. Additional observations have not supported this hypothesis.

Some false positives are undoubtedly due to mistaken identity. While most of these cases are accidental, samples of urine from known positive mares have been submitted on occasions in lieu of samples from the mares that were supposed to have been tested. Now all mares are retested before they are placed on production.

The biological reasons why false positives occur have been ably discussed by Mayer. (20) Briefly, it is supposed that abortion or resorption or the foetus are responsible. So far, we have been unable to demonstrate any other causes, although some have been investigated.

Although our data are incomplete, our experience with the Cuboni

test over a three year period would appear to indicate that it is at least 90% accurate in detecting pregnant mares after the 120th day of pregnancy.

In the Ottawa Valley this year, several thousand mares will be purchased for the purpose of collecting urine from them. Most buyers have learned by experience that all their favorite signs for diagnosing pregnancy cannot compare with the chemical test for accuracy, and have come to rely on the latter exclusively. As it takes time to ship samples and reports to and from Montreal, several inquiries have been made regarding the practicability of the local veterinarian conducting the tests. This is quite possible, and for those men who wish to undertake the work, the technique described by Cole and Hart (21) is suggested as most adaptable to existing conditions. Briefly, this is as follows:

"Acidify 5 cc. of urine with .1 cc. of concentrated hydrochloric acid. Place in a boiling water bath for 10 minutes, cool and extract with 6 cc. of benzene. Dry 3 cc. of the extract at 60 to 80 C. Add 4 cc. of concentrated sulfuric acid to the residue and warm in water bath to 70 to 80 C., and read the results in 10 minutes.

"Extracts of urine from pregnant mares will give a green fluorescence in reflected light, whereas those from non-pregnant mares will be reddish-brown. Very frequently, there will be a very slight greenish tinge in extracts from nonpregnant mares but no difficulty should be experienced in differentiating the two."

Hormonal tests for pregnancy are relatively costly and are only done because diagnosis by manual examination has apparently proven highly inaccurate. While some veterinarians are exceedingly competent at this work others are not, and on occasions pregnant mares have been aborted by rough or careless handling.

We have found that farmers are willing to pay to know whether mares are pregnant early in gestation, and as it would appear that the collection of pregnant mares' urine will become an established industry. Veterinarians located in, or near, areas where collection depots are situated, or where there is considerable horse breeding going on, would be well advised to become adept at the manual diagnosis of pregnancy.

When mares have passed the Cuboni test for pregancy, they are shipped to one of the collection depots. The size of the units varies from those containing only five or six mares to over one thousand.

In the large units, prevention of disease is a major problem. To date, relatively few serious outbreaks of distemper, influenza, or other infectious diseases have occurred. This is largely due to the measures adopted for early recognition of disease, isolation of infected animals, plus the liberal use of sulfanilamide.

Abortions of undertermined cause have been troublesome. Clinically, they resemble virus abortion, as described by Dimock. (22) With increased breeding activity, it is natural to expect the incidence of this winter to determine definitely what may be the cause of these abortions. Preliminary tests suggest that they are not due to *Salmonella abortiva equina.*

The actual collection of pregnant mares' urine offers certain technical

difficulties. At first, it was usual to collect the urine off the floor, grossly contaminated with feces and bedding. While this does not detract materially from the quality of urine from which it is intended to extract free oestrone, it has a decided detrimental effect on the water soluble conjugated oestrogens. Therefore, in the absence of technical apparatus, it was necessary to have men collect urine from the mares as it was voided. To do this job well requires a very active, agile individual, as many mares give little or no indication that they are about to urinate. As most such individuals could earn big wages in war industry, and as there was a certain social stigma associated with this work in the early days, it was extremely difficult to obtain suitable men. This led to attempts to devise any apparatus that would automatically collect the urine and exclude the feces. Such a contraption has been developed, and is now used in all units. The perfection of this harness has done a great deal to improve the quality of the urine and to simplify the labour problem which was so acute.

When the containers are filled, the contents are placed in barrels containing a suitable preservative and refrigerated to retard bacterial growth. When the barrels are filled, they are shipped to the plant where the material undergoes numerous processes before the minute quantity of active material is recovered. The percentage recovery of active material is relatively low, and while the gross quantity of urine collected is measured in hundreds of tons, the active material is measured in milligrams.

With the improvements that have been made in the collection of P.M.U., it has been possible to reduce the cost of the finished product to the public. As a result, orally active oestrogens are now available at a price that practically every patient can afford to pay. This, in return, has made necessary the collection of additional quantities of P. M. U. to satisfy the demand.

Leading gynaecologists are agreed now that there is no justification for injecting women with oestrogens once or twice weekly when an equally satisfactory response may be obtained by oral administration. Therefore, the demand for these orally active products will continue to increase, while those for parenteral use will, no doubt, diminish in popularity.

There is always the possibility that synthetic products will supplant natural sources. This is a constant source of worry to those who have thousands of dollars invested in this enterprise. However, certain important oestrogens have already been synthesized. For instance, Marker et al (23) in 1936 converted ergosterol to oestrone, while Bachman (24) succeeded in synthesizing equilenim. However, the procedures are costly, and are not likely to be perfected to the point where they can displace P. M. U.

In addition to the natural hormones, there have been a great variety of other compounds synthesized that possess oestrogenic activity. The best known of these are stilboestrol and hexoestrol. While it is generally agreed that synthetic oestrogens are effective for the relief of hot flushes associated with the menopause, at the same time the patients often experience nausea, and do not feel much better. When natural oestrogens, such as so-

dium oestrone sulphate, are given in equivalent doses, the symptoms are relieved, and, in addition, the patient feels much better. Allen (25) states that "experience with other menopause patients, especially young castrated ones, in whom the uterus was removed and who consequently could no be used for this study, seems to indicate that stilboestrol is not so effective as the natural oestrogens in relieving the unpleasant symptoms of the menopause. It likewise certainly produces nausea in many patients when 0.5 mg or more per day is given. The natural oestrogens, however, can be given in several times the equivalent dose without the production of any nausea". In human medicine, this extra feeling of well-being that is experienced by patients following administration of natural oestrogens is exceedingly important. Women will almost invariably pay a cent or two more for a product that will relieve their symptoms, and, at the same time, make them feel much-better, than they will for a cheaper product that simply relieves their symptoms but does not give them the important feeling of well-being.

In veterinary medicine, subjective symptoms, such as nausea, are not given serious consideration. In animal practice, cost is the most important factor, and what the patient feels like is of secondary importance. Thus, while natural oestrogens have proven much more acceptable to the medical profession, synthetic oestrogens, such as diethylstilboestrol dipropionate, are used almost exclusively in veterinary practice, not because they are better than natural oestrogens but simply because they are cheaper. Of the synthetic oestrogens now available, diethylstilboestrol dipropionate would appear to be the most suitable. As stated by Rowlands, (26) "the optimal rate of absorption of stilboestrol is procured when the substance is combined with propionic acid to form the dipropionate", and clinical experience would appear to support this statement.

Oestrogens appear to have many indications in veterinary medicine. Because of their marked effect on the uterus, increasing its water content, stimulating proliferation of the muscular and epithelial cells, increasing uterine motility and secretion, they have been widely used not only to initiate oestrus but as aids in the treatment of retained placenta and endometritis in all species of animals. They have also been suggested for the treatment of incontinence in spayed bitches, false pregnancy and for benign prostatic hypertrophy.

Reece, (27) Barker (28) and others have demonstrated that it is possible to stimulate the production of economic quantities of milk by well developed, nonpregnant heifers following treatment with diethystilboestrol dipropionate. The dosage generally suggested is 20 mg. twice weekly for 8 to 12 weeks, or until engorgement of the udder occurs, when milking should be commenced. There is some evidence that placing a calf with the cow may be a more effective stimulus to milk secretion than manual or machine milking.

Recently, unconfirmed reports have been received that 40 to 50 mg. of diethylstilboestrol dipropionate injected twice weekly will stimulate milk secretion in nonlactating, nonpregnant adult cows. It must be emphasized,

however, that stilboestrol or other oestrogens do not stimulate milk secretion in lactating animals.

Synthetic oestrogens have also been found useful in preparing fowl for market. Lorenz (29) has shown that subcutaneous implantation of diethylstilboestrol pellets (15 mg. to 20 mg.) 4 to 5 weeks before birds are to be marketed results in more rapid increases in fat deposition. The muscle meat of old, tough birds is also said to be tenderized and lightened in colour. As the pellets are relatively inexpensive and readily administered, their use in practical poultry husbandry may be expected.

Jaap and Thayer (30) have experimented with the oral administration of oestrogens to birds to rapidly increase fat deposition. Diethylstilboestrol powder at rate of 23 mg. per lb. of feed was ineffective, while larger quantities were too expensive. Ethinyl oestradiol gave a satisfactory response but was too expensive for practical use. Of the various oestrogens tested by them, the dimethyl ether of diethylstilboestrol appeared most satisfactory from both a performance and cost point of view.

With the increased use of oestrogens, the danger of toxic reactions occurring following administration of large or continued doses must be considered. However, the likelihood of permanent damage following such use appears to be slight. Allen and Diddle (31) were unable to show that any permanent damage occurred following prolonged administration of oestrogens to monkeys. Similarly, it has been noted that heifers that are administered large and frequent doses conceive after milk secretion has commenced.

Oestrogens have established a definite place for themselves in human and veterinary medicine and it appears that pregnant mares' urine will continue to be the chief commercial source of the natural product for many years to come.

From the humble beginning a few short years ago when only ten or fifteen mares were on production in Canada, the demand has increased until now many thousand mares are being utilized and this year upwards of a million dollars will be paid to farmers in Quebec and Ontario for the raw material.

All of the oestrogen manufactured in Canada from this source is not required to meet our domestic demand. In fact, the bulk of the material is exported, a large portion going to the United States, but sizeable quantities also to Brazil, Argentina, Egypt and the British West Indies. With the cessation of hostilities, it is expected that additional amounts will be required for the European market.

The development of the oestrogen-production industry has done a great deal to assist agriculture in Canada. It remains to be seen whether the Canadian industry that has been built up will be able to withstand in later years competition from those Central European countries which formerly supplied a considerable portion of the oestrogen requirements of the Western hemisphere.

The abundance of suitable land and horses, the perfection of semi-automatic collection equipment; efficient means of transportation and

methods of processing, suggest that the Canadian oestrogen-production industry will be able to operate economically and permit the finished product to compete favourably in the world market.

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